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Efficient stem cell isolation from under vacuum preserved tissue samples

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Abbreviations: UVSC, under vacuum sealing and cooling; FBS, foetal bovine serum; THP, Tamm-Horsfall protein; H/E, hematoxylin/eosin; EBM, endothelial basal medium

Different approaches for the isolation of stem/progenitor cells have been reported, including stem cell selection in stringent culture conditions. We evaluated the possibility of isolating human progenitor cells from surgical specimens preserved by under vacuum sealing and cooling, a clinical practice approached by several hospitals as alternative to formalin. Renal tissue samples (n = 20) maintained under vacuum from 6 to 48 h at 4°C were used to isolate human renal CD133$^+$ progenitor cells. We obtained CD133$^+$ progenitors from unsorted cells derived from disaggregated tissues from each sample. Phenotypic characterization as well as in vitro and in vivo differentiation of the obtained CD133$^+$ lines showed results comparable with sorted CD133$^+$ cells obtained from fresh tissue. These results indicate that the process of sealing under vacuum and cooling appears as a suitable tissue treatment to isolate hypoxia resistant cells, such as human stem/progenitor cells, and that this procedure can be exploited to render the extraction of stem cells from human samples more practical and feasible.

Introduction

Isolation and culture are crucial techniques for studying stem cell biology and modulation. In particular, in studies on human cells, samples from tissue removed for clinical procedures and discarded by pathologists are commonly utilized as a stem/progenitor source for research studies. However, the routine use of formalin, both as a preserver and fixative for histological processing, may limit the possible use of pathological samples for cell isolation. As formalin is encountering increasing criticisms for toxicity, carcinogenicity and environmental concerns,[1] several hospitals are now approaching the use of fresh tissue sample transfer from surgery to the pathology service.[2] Such transfer, and related "ischemic time," is heavily dependent on local conditions and habits.

In the major university hospital we are related to, transfer of surgical specimens under condition of vacuum sealing and cooling (UVSC) has become a habit for the past 4 years.[2] Merits of this procedure in terms of morphological, immunohistochemical and nucleic acid preservation have already been reported.[3] We have considered that the UVSC procedure may offer advantages for stem cell preservation and culturing as well. In fact, low oxygen tension is an important component of the stem cell microenvironment and niche and it provides signals conducive to the maintenance of definitive stem cell properties.[4,5] We therefore hypothesized that the anoxic conditions of tissue samples under vacuum may allow survival of undifferentiated stem/progenitor cells.

We previously reported on the isolation of CD133$^+$ progenitor cells from normal fresh specimens of human kidney.[6] In the present study, we show the successful isolation of CD133$^+$ cells from 20 renal tissue samples maintained under vacuum from 24 to 48 h at 4°C. The results show in all cases a selective survival of stem cells in the anoxic condition characterizing the vacuum procedure, and show that this approach is suitable for stem cell isolation in terms of feasibility and practice.

Results and Discussion

Different approaches for the isolation of stem/progenitor cells have been reported. A direct method may involve the isolation of stem cells by a known marker, such as CD133,[7] or alternatively by a cell function such as the capacity to efflux Hoechst dye.[8] Negative methods are based on the elimination of unwanted differentiated or contaminating cells. In this regard, selective culture conditions that only allow survival of undifferentiated cells might be used, e.g., by removal of serum and by using plastic dishes that do not support cell adhesion.[9,10] Here, we evaluated UVSC treatment of normal tissues could be useful for selective survival and isolation of human renal CD133$^+$ progenitor cells.

CD133$^+$ progenitor cells are present as a minor population within the renal tubules of the nephron and were previously isolated by immunomagnetic sorting.[6,11] As in tissue undergoing vacuum and cooling the percentage of viable cells was very low

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(ranging between 11% to 27% cells, n = 5 experiments), we plated the entire renal population in culture dishes, in an attempt to discover whether the hypoxia could select for renal progenitors (**Fig. 1**).

Ischemia time in tissues undergoing UVSC between surgical removal and arrival at the pathology laboratory, from where we collected the specimens for cell culture, ranged between 6 and 48 h (mean 28.8 ± 10.4 h). Samples were discarded if subjected to > 48 h vacuum. Cells were plated in complete endothelial basal medium (EBM) without fetal bovine serum (FBS) at a density of 1.0×10^4 cells per cm^2. The unattached cells were removed after 72 h, and few cells were observed in the flasks, which in the subsequent days generated colonies (**Fig. 2A**). The confluence was typically reached after 7–10 d of culture (**Fig. 2A**) and cell lines were obtained in all different tissue samples used for isolation. Twenty different lines were obtained and characterized by fluorescence-activated cell sorting analysis.

The obtained CD133$^+$ cell lines derived from the under-vacuum tissues (UVSC CD133$^+$ cells) showed CD133 expression > 90% cells in all cell lines (**Fig. 2B**). No contamination of endothelial cells of inflammatory cells was shown by evaluation of CD31, CD34, CD117 and CD45 markers, which remained almost undetectable (**Fig. 2B**). In addition, UVSC CD133$^+$ cells expressed the mesenchymal markers CD29, CD73 and CD44 (**Fig. 2B**) and, as evaluated by immunofluorescence, vimentin (**Fig. 3A**). Cytokeratin was also expressed by undifferentiated cells (**Fig. 3A**). This phenotype was super-imposable to that of sorted CD133$^+$ cells.[6,11] In addition, UVSC CD133$^+$ cells expressed the renal specific transcription factor PAX2, known to be involved in renal embryogenesis[12] as well as stem transcription factors associated with multipotency,[13] OCT4A, c-MYC and KLF4, at levels comparable to those of sorted CD133$^+$ cells (**Fig. 2C**). Three different UVSC CD133$^+$ cell lines were subjected to clone generation by limiting dilution technique in 96 well plates. A total of 107 clones were collected, with a cloning efficiency equal to 37.1 ± 9%, that did not differ to that reported for sorted CD133$^+$ cells.[6] Eleven clones were expanded up to 1×10^7 cells. Cytofluorimetric analysis showed expression of CD133$^+$ on > 90% cells in all clones, as well as of other mesenchymal markers (not shown), indicating that their phenotype was super-imposable to that of the originating cell line.

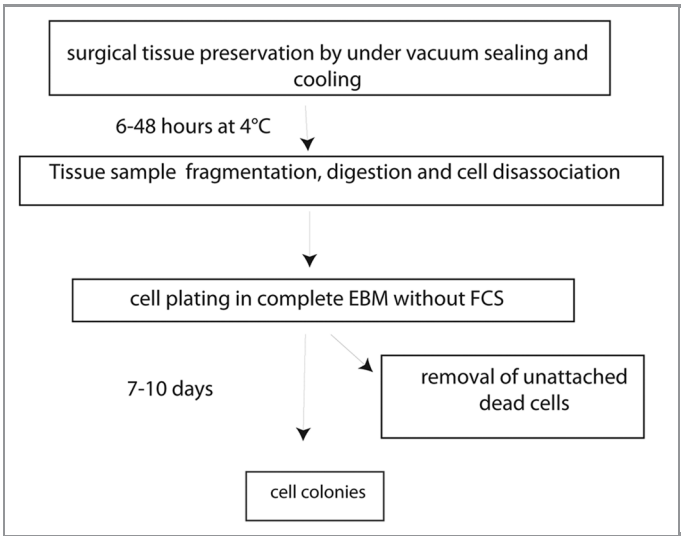

Figure 1. Schematic representation of the consequential steps for the isolation of human renal CD133$^+$ cells from tissues preserved by UVSC.

In vitro UVSC CD133$^+$ cultured in differentiating medium showed the ability to undergo epithelial differentiation, as shown by loss of vimentin (**Fig. 3A**) and reduction of PAX2 (**Fig. 2D**). In addition, differentiated cells acquired markers characteristic of fully differentiated renal epithelia, such as megalin, mainly expressed by proximal tubular epithelial cells[14] and Tamm-Horsfall (THP), expressed by the ascending limb of the loop of Henle and distal convolute tubules (**Fig. 3A**).[15] In vivo, undifferentiated UVSC CD133$^+$ cells (1×10^6) were subcutaneously injected into Matrigel in severe immunodeficient (SCID) mice. After 15 d, cells spontaneously organized into elongated epithelial tubular structure with aspects of cuboidal or flat epithelium (**Fig. 3B**). Immunohistochemical evaluation indicated that these structures were human, as they expressed HLA class I antigen (**Fig. 3B**, inset). Similar results were obtained for sorted CD133$^+$ cells.[6,11] Whereas in vitro the majority of the differentiated cells expressed marker of both proximal tubules (megalin) and of the loop of Henle (THP) (**Fig. 3A**), the differentiated structures originated in the in vivo experiments presented a selective expression of nephron markers (**Fig. 3B**). In particular, the proximal tubule marker amino peptidase A[16] was expressed by structure with a cuboidal epithelium and not by those expressing flat epithelium (**Fig. 3B**). In analogy, the THP expression was restricted to a segment of a tubular structure generated in Matrigel, that morphologically resembled the loop of Henle (**Fig. 3B**). These observations suggest the possibility that a tri-dimensional organization is required for the complete differentiation of CD133$^+$ cells into segment-specific cells of the nephron.

The results show that samples in UVSC can be used for stem/progenitor cell isolation. It can be speculated that the hypoxic conditions present in the tissue after vacuum treatment may select for progenitor cells, which are generally more resistant.

The UVCS procedure may offer several advantages for progenitor/stem cell culture. The first advantage is the increase in the number of samples suitable for cell isolation. In this regard, as tissue sampling of fresh tissues for cell culture must be performed before formalin fixation, it is generally performed in the surgery service. The possibility to use the discarded tissues from the pathology service has advantages for the correct evaluation of the pathological sample by the pathologist. In addition, tissues can be used 24–48 h after removal, limiting the need of a prompt presence of the cell biologist in the surgical service. Finally, the UVCS procedure leads to selection of hypoxia-resistant cells, and therefore may limit the contamination of unwanted cell types or of differentiated cells within the culture. In contrast, a clear disadvantage of the UVCS procedure is the loss of other cell populations when needed.

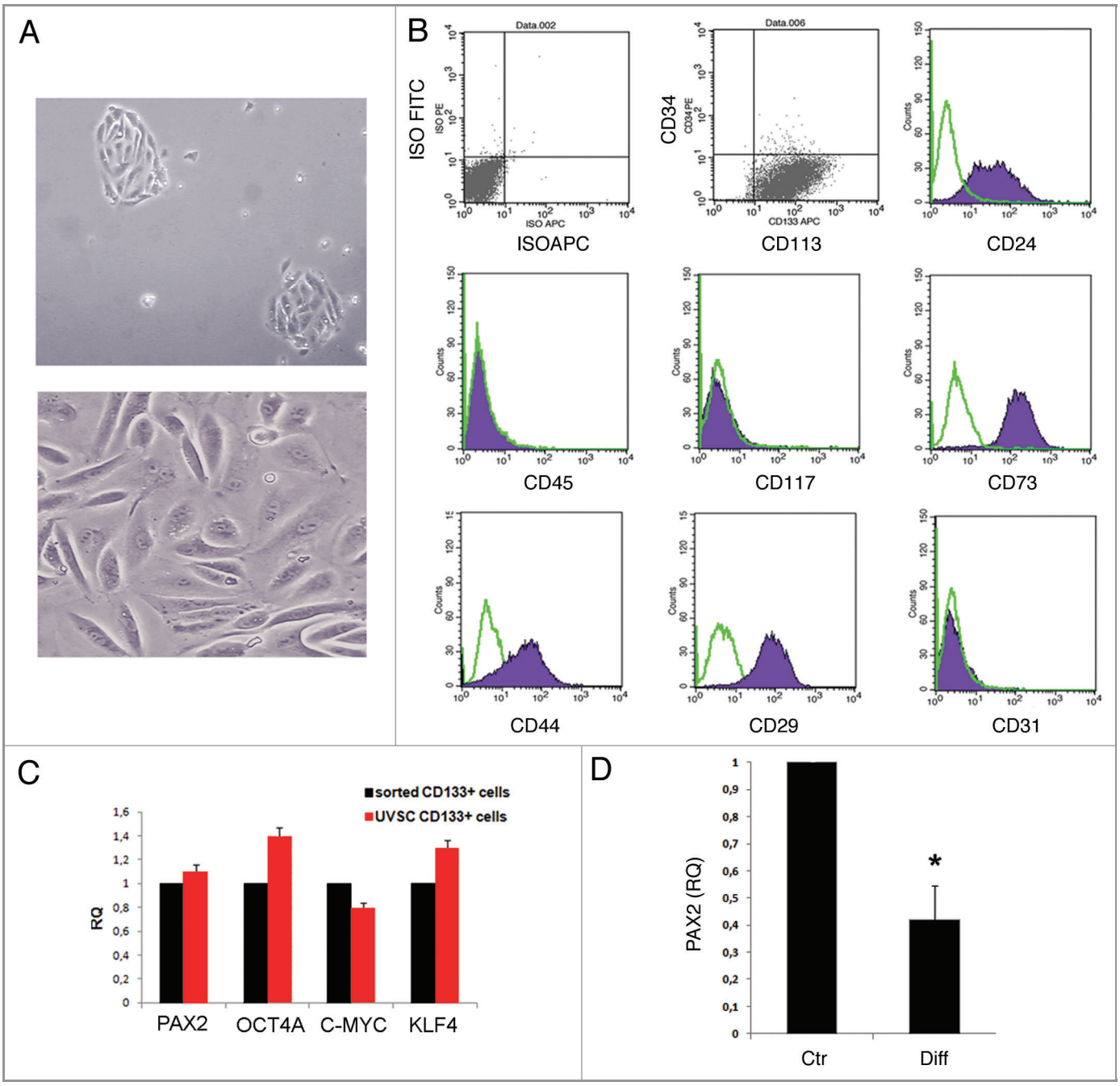

Figure 2. Characterization of human renal UVSC CD133$^+$ cells. (**A**) Morphology of cells isolated from tissues preserved by UVSC after 5 d (top) and 10 d (bottom) culture. Original magnification 40×. (**B**) Representative FACS analyses of UVSC CD133$^+$ cells. In the lower histograms, the filled area shows binding of the specific antibody and the green line of the isotypic control. All 20 lines showed similar marker expression. (**C**) Quantitative RT-PCR analysis of UVSC CD133$^+$ cells or of CD133$^+$ cells sorted by fresh tissue (sorted CD133$^+$ cells) showing the expression of mRNAs encoding for the renal embryonic and stem-cell related transcription factors. All data were normalized to β-actin mRNA. The mean of five different cell lines was normalized to 1 for sorted CD133$^+$ cells. Student's t test showed no statistical difference. (**D**) Quantitative RT-PCR analysis of UVSC CD133$^+$ cells in standard culture condition (Ctr) or after epithelial differentiation (Diff), showing the expression of mRNAs encoding for the renal embryonic transcription factor PAX2. All data were normalized to β-actin mRNA. The mean of two different cell lines tested in triplicate was normalized to 1 for Ctr. Student's t-test: *$p < 0.05$.

In conclusion, UVCS procedure appears as a suitable tissue treatment to obtain hypoxia resistant cells, such as human stem/progenitor cells, in culture and is can be exploited to make the extraction of stem cells from human samples more practical and feasible.

Materials and Methods

Vacuum tissue treatment. A semi-professional machine (VAC 10, Milestone, www.milestonemedsrl.com) capable of handling large bags was used for the procedure. Tissues immediately after

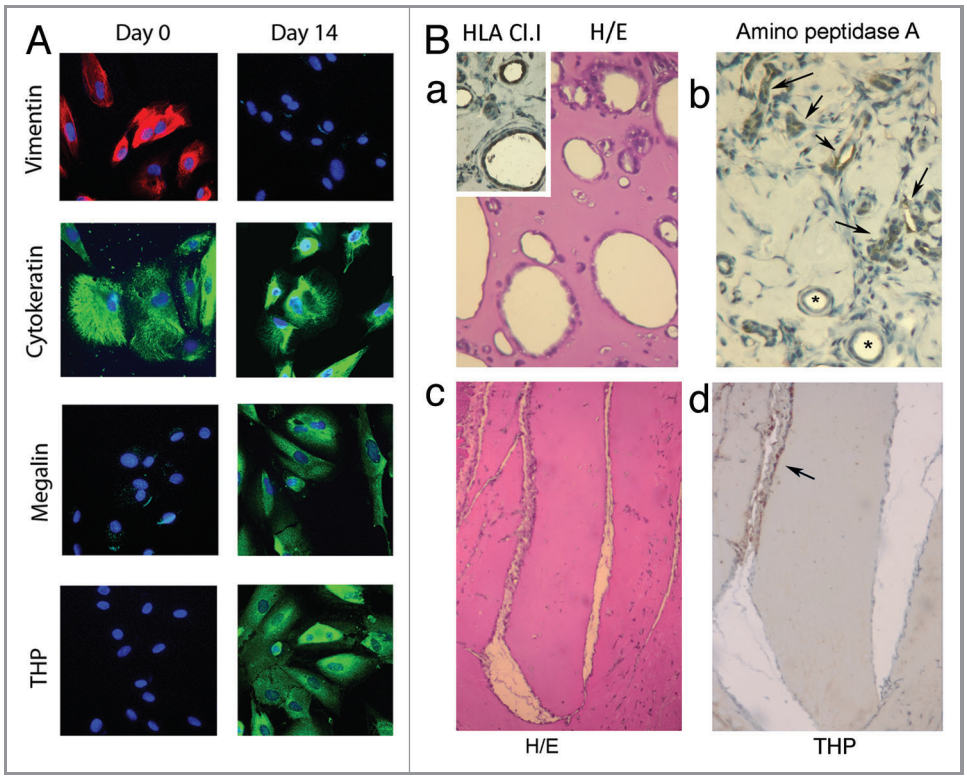

Figure 3. In vitro and in vivo differentiation of human renal UVSC CD133⁺ cells. (**A**) Representative micrographs showing the immunofluorescence staining of UVSC CD133⁺ cells for mesenchymal and nephron markers before (day 0) and after (day 14) culture in epithelial differentiating medium. Nuclei were stained with Hoechst dye 33342. Original magnification 630×. Four different experiments were performed with similar results. (**B**) Undifferentiated UVSC CD133⁺ cells (1×10^6) were injected subcutaneously with Matrigel in SCID mice and plugs recovered after 14 d. Representative hematoxylin/eosin (H/E) stained micrographs (**a and c**) show tubular-like elongated structures covered both by cuboidal and by flat cells. By immunohistochemistry, structures were positive for human HLA class I (**a**, inset). Structures with cubical epithelium were stained by the proximal tubule marker amino peptidase A (**b**, arrows) whereas structures with flat epithelium were negative (**b**, star). The Henle's loop thick limb marker THP selectively stained a segment of the elongated tubular structure originated from UVSC CD133⁺ cells (**d**, arrow). Original magnification 250×. Four different cell lines were analyzed with similar results.

removal were put in plastic bags with or without an identification label (which can alternatively be attached on the surface of the bag). Vacuum and sealing processes took approximately 15 sec. The specimen were then kept in a local fridge at 4°C until transfer to the pathology lab for reduction. In case of urgency operations during the weekend, the under-vacuum tissues were kept at 4°C up to 48 h.

Cell isolation and culture conditions. UVSC CD133⁺ cells were obtained from the normal portion of the inner medulla obtained from surgically removed kidneys for polar carcinomas (17/20) or for benign pathologies (reflux nephropathy, n = 2 and chronic calculus disease, n = 1), after approval of the ethical committee for the use of human tissue of the University of Torino. Tissue samples were cut to obtain 3–5 mm^3 fragments and digested in 0.1% Collagenase type I (Sigma-Aldrich) for 45 min at 37°C. Tissue was subsequently forced through a graded series of meshes to separate the cell components from stroma and aggregates. Cells were re-suspended in expansion medium [endothelial basal medium (EBM) plus supplement kit; Cambrex BioScience] without serum addition at a density of 1.0×10^4 viable cells per cm^2. UVSC CD133⁺ cells were compared with CD133⁺ cells isolated by fresh tissue by magnetic cell sorting, using the MACS system (Miltenyi Biotec), as described.[6] To assess their capacity to generate colonies cells were seeded using a limiting dilution technique in a 96 well plates. After 12 h, wells not containing single cells were discarded by microscopical visualization and clones derived from a single cell were routinely observed and expanded in expansion medium.

Cytofluorimetric characterization. Flow cytometry was performed as described.[6] The following monoclonal antibodies (mAb) were used, all fluorescein isothiocyanate or phycoerythrin-conjugated anti-CD133/1 (clone AC133) (clone 293C3, Miltenyi Biotech GmbH); anti-CD24, -CD29, -CD31, CD117, -CD34, -CD44, -CD45, -CD73 and -CD90 (all from Becton Dickinson). Fluorescein isothiocyanate or phycoerythrin-conjugated mouse nonimmune isotypic IgG (Miltenyi Biotech) were used as negative controls.

Quantitative real-time PCR. For gene expression analysis, quantitative real-time PCR was performed as previously described.[17] Briefly, real-time PCR experiments were performed in 20 µl reaction mixture containing 5 ng of cDNA template, the sequence-specific oligonucleotide primers (purchased from MWG-Biotech AG, www.mwg-biotech.com) and the Power SYBR® Green PCR Master Mix (Applied Biosystems). β-actin or

TATA binding protein (TBP) mRNA were used to normalize RNA inputs. Fold change expression with respect to control (sorted CD133$^+$ cells) was calculated for all samples. The following sequence-specific oligonucleotide primers were used: human OCT4A: forward, 5'-AGC AGG AGT CGG GGT GG-3' (nt 348–364) and reverse, 5'-CTG GGA CTC CTC CGG GTT-3' (nt 465–448); human KLF4: forward, 5'-CCA TTA CCA AGA GCT CAT GCC-3' (nt 1683–1703) and reverse, 5'-GGG CCA CGA TCG TCT TCC-3' (nt 1761–1744); human c-MYC: forward, 5'-CAG CGA CTC TGA GGA GGA ACA-3' (nt 1316–1336) and reverse, 5'-TGA GGA GGT TTG CTG TGG C-3' (nt 1444–1426); human PAX-2: forward, 5'-CCC AGC GTC TCT TCC ATC A-3' (nt 938–956) and reverse, 5'-GGC GTT GGG TGG AAA GG-3' (nt 1002–986); human TBP: forward, 5'-TGT GCA CAG GAG CCA AGA GT-3' (nt 938–957 iso1) and reverse, 5'-ATT TTC TTG CTG CCA GTC TGG-3' (nt 988–968 iso1) and human β-actin: forward, 5'-TGA AGA TCA AGA TCA TTG CTC CTC-3' (nt 1058–1081) and reverse, 5'-CAC ATC TGC TGG AAG GTG GAC-3' (nt 1151–1131).

In vitro differentiation. Epithelial differentiation was done by culturing cells for 10 d in expansion medium with 10 ng/ml human hepatocyte growth factor (HGF; Sigma Aldrich) and 10 ng/ml human FGF-4 (Sigma-Aldrich).[2] Indirect immunofluorescence was performed on cells cultured on chamber slides as described,[6] using the following antibodies: anti-pan-cytokeratin mAb (Immunological Sciences), anti-THP, anti-human HLA-Class I, anti-megalin, rabbit Abs (all from Santa Cruz Biotechnology) and anti-vimentin mAb (Sigma). Recognition of primary antibodies was done using Alexa Fluor 488 or Texas Red conjugated anti-rabbit, anti-mouse or anti-goat antibodies (Molecular Probes).

In vivo differentiation. UVSC CD133$^+$ cells were implanted subcutaneously into SCID mice (Charles River, Jackson Laboratories,) within Matrigel (Becton Dickinson), as described,[6] in adherence to the Italian recommendations for the care of laboratory animals (1×10^6 in 250 µl of Dulbecco's modified Eagle's medium plus 250 µl of Matrigel at 4°C). Cells were injected subcutaneously into the left back of SCID mice via a 26-gauge needle using a 1-ml syringe. At day 15 d, mice were sacrificed and Matrigel plugs recovered and analyzed. Sections from paraffin-embedded blocks of human CD133$^+$ cells placed in Matrigel in SCID mice were collected onto poly-L-lysine-coated slides. Endogenous peroxidase activity was blocked with 6% H_2O_2 for 8 min at room temperature. Primary antibodies (anti-human HLA Cl.I, anti-amino peptidase A and anti-THP polyclonal rabbit antibodies from Santa Cruz Biotechnology) were applied to slides overnight at 4°C. The following antibodies were used: horseradish peroxidase-labeled anti-rabbit Envision polymers (Dako) were incubated for 1 h. The reaction product was developed using 3,3-diaminobenzidine. Omission of the primary antibody or substitution with an unrelated rabbit serum or mouse IgG served as negative control.

Statistical analysis. Statistical analysis was performed by using the Student's t-test. A p value of < 0.05 was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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